Algal Biomass for Bioenergy and Bioproducts Production in Biorefinery Concepts

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Publication date:
2017

Document Version
Publisher's PDF, also known as Version of record

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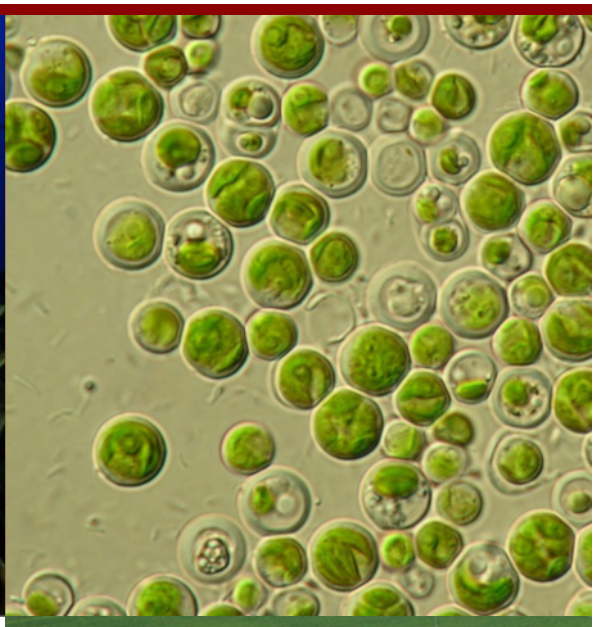
PhD Thesis



Martina D'Este, June 2017

DTU Environment
Department of Environmental Engineering

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The synopsis part of this thesis is available as a pdf-file for download from the DTU research database ORBIT: http://www.orbit.dtu.dk.

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Preface

This PhD thesis, entitled "Algal Biomass for Bioenergy and Bioproducts Production in Biorefinery Concepts", comprises the research carried out at the Department of Environmental Engineering, Technical University of Denmark from January 01, 2014 to December 31, 2016. Professor Irini Angelidaki and researcher Merlin Alvarado-Morales were supervisor and co-supervisor, respectively.

The thesis is organized into two parts: the first part puts the findings of the PhD into context in an introductive overview; the second part consists of the papers and scientific reports listed below. These will be referred to in the text by their paper number written with the Roman numerals I-V.

Papers:

I D'Este M., Alvarado-Morales M., Ciofalo A., Angelidaki I., 2017. Macroalgae *Laminaria digitata* and *Saccharina latissima* as potential biomasses for biogas and total phenolics production; focusing on seasonal and spatial variations of the algae. (Accepted for publication in Energy & Fuels)

II D'Este M., Alvarado-Morales M., Angelidaki I., 2016. Amino acids production focusing on the fermentation technologies – A review. (Under review in Biotechnologies Advances)

III D'Este M., Alvarado-Morales M., Angelidaki I., 2017. *Laminaria digitata* as potential carbon source in heterotrophic microalgae cultivation for the production of fish feed supplement. (Under review in Algal Research)

IV D'Este M., De Francisci D., Angelidaki I., 2017. Novel protocol for lutein extraction from microalga *Chlorella vulgaris*. (Under review in Biochemical Engineering Journal)

V De Francisci D., D'Este M., Rasouli Z., Angelidaki I., 2017. Novel biorefinery concept for the extraction of lutein and proteins from microalga *Chlorella vulgaris* and generation of biogas from the residual biomass. (Submitted to Bioresource Technology)

Scientific Report:

I D'Este M., Rodríguez M. G., Alvarado-Morales M., Angelidaki I., 2016. Integration of carbon dioxide fixation by microalgae grown in biogas with single-cell protein production.

In addition, the following publications, not included in this thesis, were also concluded during this PhD study:

I Nielsen M. M., Manns D., D'Este M., Krause-Jensen D., Rasmussen M. B., Larsen M. M., Alvarado-Morales M., Angelidaki I., Bruhn A., 2016. Variation in biochemical composition of *Saccharina latissima* and *Laminaria digitata* along an estuarine salinity gradient in inner Danish waters. Algal Research 13, 235-245.

II Seghetta M., Romeo D., D'Este M., Alvarado-Morales M., Angelidaki I., Bastianoni S., Thomsen M., 2017. Seaweed as innovative feedstock for energy and feed - evaluating the impacts through a Life Cycle Assessment Paper. Journal of Cleaner Production 150, 1-15.

III Van Wagenen J., Pape M. L., Safafar H., D'Este M., De Francisci D., Angelidaki I., Photobioreactor design and operation influences biochemical composition of waste-grown microalgae. (Submitted)

Acknowledgements

This thesis is the result of three years of adventure at DTU Environment where I had the chance to develop myself both personally and professionally.

First of all, I would like to thank my supervisor Professor Irini Angelidaki for giving me the opportunity to be a PhD student here and for her constant guidance, contagious enthusiasm and ambitious ideas during the last three years.

I must also thank my co-supervisor Dr. Merlin Alvarado-Morales for inspiring me when needed, for his supervision and support.

Moreover, I want to thank Mikael Emil Olsson and Satomi Matsura for their availability and support in the lab and Marie Kampmann Eriksen for the Danish translation of the summary.

A special thanks goes to the “mum” of all the PhD students in our department, Anne Harsting. Thanks a lot for the constant secretarial and personal assistance during these three years.

Finally, I want to thank my friends inside and outside DTU and all my colleagues at DTU that have made my time here so special.

And, last but not least, a super big thanks goes to my parents and my brother. Even if from far away, they were constantly by my side during this Danish adventure, making possible for me to pass through the struggles of this PhD. Without them all of this would not be possible and I will always be grateful for all the support that they gave me throughout my life. This is why I want to dedicate this thesis to them and to my grandmother who is always protecting me from the heaven.

Abstract

The fast population growth is increasing the demand for energy and resources. However, the reserves of oil are diminishing and greenhouse emissions associated to its combustion are affecting the global climate causing global warming. Therefore the need for alternative resources and processes is becoming impellent.

Macro- and microalgae have the ability to transform nutrients into valuable biomass. Being a good source of vitamins, minerals, lipids, proteins and pigments, they represent a promising source of various products. However these biomasses are still very little explored as biorefinery feedstocks.

Biorefinery represents an important tool towards the development of a sustainable economy. Within the biorefinery framework several bioproducts, such as food, feed and biofuels, can be produced from biomass. The specific composition of the biomass feedstock determines the potential final product that can be obtained.

In this thesis, micro- and macroalage were investigated as biorefinery feedstocks. The main aim of this work was developing different biorefinery strategies for the production of high value products, such as proteins or pigments, to be employed in the pharmaceutical or nutraceutical industry. The macroalgae used in this work were *Laminaria digitata* and *Saccharina latissima*, while the microalgae were *Chlorella sorokiniana*, *Chlorella vulgaris* and *Chlorella protothecoides*.

Moreover, an evaluation of the effect of the harvesting season and location on the composition of high value products such as total phenolics and on the biogas potential for *L. digitata* and *S. latissima* was done. Both these factors had a significant impact on the accumulation of total phenolics in the algal biomass and on the biogas production. In particular, samples harvested in summer, because of the high content of sugars, showed to be the most

promising feedstock in the development of biorefinery processes, containing 0.5 mgTPC gDM^{-1} and having a biomethane potential of 343.7 $NmLCH_4$ g VS^{-1}.

Moreover, proteins being an interesting valuable product to be used as food and feed supplement, diverse industrial methods to produce amino acids and proteins were analyzed. Innovative techniques to increase the protein content in the final biomass, such as microalgae or microorganisms to be used as single cell proteins (SCP), were also investigated. The combination of phototropic growth of *C. sorokiniana* with *Methylococcus capsulatus* led to an innovative solution where two products rich in proteins (up to 43 %DM) were obtained.

Another strategy developed in this thesis work was based on the combination of micro- and macroalgae to enhance protein production. Indeed, the microalgae *C. protothecoides* was grown heterotrophically in the macroalgae *L. digitata* hydrolyzed. The final composition of the microalgal biomass showed that the protein content was increased from 0.07 ± 0.01 gProtein gDM^{-1} to 0.44 ± 0.04 gProtein DM^{-1}. The results obtained show that this solution may represent an interesting strategy to be applied in a biorefinery approach.

Finally, a microalgae biorefinery strategy was developed. Lutein represents a very important pigment present in the macular region of the human eye. It is crucial in the protection against light-induced retinal damages and responsible for maintaining human bone health and preventing some diseases. Lutein and proteins were extracted by developing innovative methods specifically designed for microalgae species. From the initial algal biomass were extracted 0.8 ± 0.1 mg Lutein gDM^{-1} with a purity of 92.5 ± 1.2% and a calculated yield of 95%. Moreover, the final protein content in the fraction was 82.7 ± 3.1% w $w^{-1}$with a protein yield of 55%. Finally, from the residues

of this extraction processes, 372.7 ± 19.0 $NmLCH_4$ gVS^{-1} of biogas were produced.

The results obtained in this thesis work show that macro- and microalgae are promising biomasses for the development of the future biorefineries.

Dansk sammenfatning

Den stigende befolkningstilvækst øger efterspørgslen efter energi og ressourcer. Oliereserverne mindskes dog, og drivhusgasemissionerne fra forbrændingen af fossile brændstoffer påvirker det globale klima og forårsager global opvarmning. Behovet for alternative ressourcer og processer er derfor stigende.

Makro- og mikroalger har evnen til at omdanne næringsstoffer til værdifuld biomasse, som er en god kilde til vitaminer, mineraler, lipider, proteiner og pigmenter. Biomassen repræsenterer derfor en lovende ressource for forskellige produkter, men er dog meget lidt udforsket som råmateriale til bioraffinaderier.

Bioraffinaderier er et vigtigt redskab i udviklingen af en bæredygtig økonomi og inden for rammerne af bioraffinaderikonceptet kan flere bioprodukter så som mad, foder og biobrændstof produceres fra biomasse. Den specifikke sammensætning af det biologiske råmateriale afgør det potentielle produkt.

I denne afhandling er mikro- og makroalger undersøgt som råmateriale til bioraffinaderi. Hovedformålet med dette arbejde er at udvikle forskellige bioraffinaderiordninger til produktion af proteinrige produkter, der skal anvendes som fødevarer og foderkosttilskud. Makroalgen anvendt i dette arbejde er Fingertang og *Saccharina latissima*, mens mikroalgeen er *Chlorella sorokiniana*, *Chlorella vulgaris* og *Chlorella protothecoides*.

Derudover er effekten af høstsæsonen og placeringen på sammensætningen af højværdiprodukter, så som fenoler, og på biogaspotentialet for *L. digitata* og *S. latissima* blevet evalueret. Begge disse faktorer har en betydelig indvirkning på produktionen af de samlede fenoler og biogas. Særligt prøver høstet i sommeren, på grund af det høje indhold af sukker, repræsenterer det mest lovende råmateriale i udviklingen af en bioraffinaderi-proces.

Forskellige industrielle metoder til at producere aminosyrer præsenteres herefter. Endvidere analyseres innovative teknikker til at øge proteinindholdet i den endelige biomasse, såsom mikroalger eller mikroroganismer, der skal anvendes som enkelte celleproteiner (SCP). Kombinationen af fototropisk vækst af *C. sorokiniana* med *M. capsulatus* fører til en innovativ løsning, hvor der kan opnås to produkter rige på proteiner.

En anden strategi er udviklet i denne afhandling, baseret på en kombination af mikro- og makroalger. Faktisk blev mikroalgerne *C. protothecoides* dyrket heterotrofisk i makroalgen L. digitata hydrolyseret. Den endelige sammensætning af mikroalgalbiomassen viste, at proteinindholdet blev forøget fra 0,07 ± 0,01 g Protein gDM^{-1} til 0,44 ± 0,04 g Protein DM^{-1}. Derfor viser de opnåede resultater, at denne løsning kan udgøre en interessant strategi, der skal anvendes i en bioraffineringsmetode.

Endelig er en mikroalge-bioraffinaderistrategi blevet udviklet. Lutein repræsenterer et meget vigtigt pigment som er til stede i det makulære område af det menneskelige øje. Det er afgørende for beskyttelse mod lysinducerede retinale skader og ansvarlig for at opretholde menneskers knoglesundhed og forebygge visse sygdomme. Lutein og proteiner produceres med innovative metoder specifikt designet til mikroalgearter. Biogas bliver desuden produceret fra resterne af denne ekstraktionsprocesser.

Resultaterne af denne undersøgelse viser at makro- og mikroalger er lovende biomasse i udviklingen af de fremtidige bioraffinaderier.

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1 Introduction

1.1 Background

The growing world population represents one of the most complex and important challenges of the 21st century. Over the last 80 years the world population has increased substantially with different growth rates in different world regions (Figure 1).

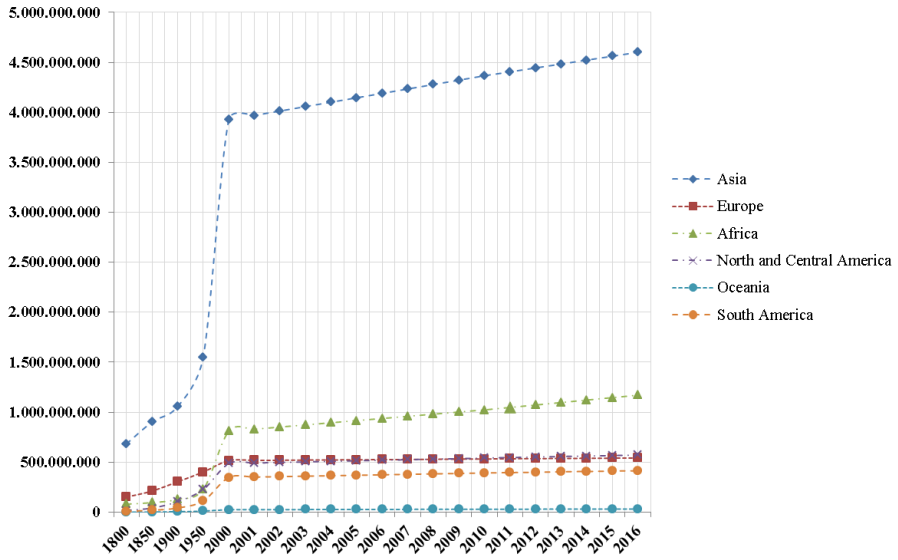

Figure 1: Growing of population by world regions from 1840 to 2016

The population growth leads to an increasing consumption of energy and resources (Hannon et al., 2010). In particular, in order to satisfy the needs of all the people in the world the energy generation and global food production have to be increased by 60% by 2050 (FAO, 2016). At present, most of the countries in the world are strongly dependent on oil for energy, chemicals and materials (Cherubini, 2010). However, the price of oil is high and its reserves are diminishing (Hannon et al., 2010). Moreover, there is scientific evidence that the greenhouse emissions associated with the combustion of petroleum derivatives, the chemical production processes and the overexploitation of land associated with human activities, are affecting the

climate and causing global warming (Loarie et al., 2009). As a result, the society is becoming aware of the need for a sustainable economy that is independent of oil (Pickett et al., 2008).

Reducing the greenhouse effect and producing energy from non-fossil sources, together with the need of securing food for all the population are key points in a sustainable development approach. Energetic policies should endorse the sustainability principles establishing international climate and agricultural directives. For this reason EU set mandatory targets to be achieved by 2020: a cut of CO_2 emissions by 20% from 1999 to 2020, 20% of the energy produce in 2020 should derive from renewable sources and a minimum target of 10% to be achieved for share of biofuels in overall EU transport gasoline and diesel consumption.

Denmark set one of the most ambitious goals among the European countries. By 2050 it aims to be fossil fuels free and to reduce by 50% its energy consumption (Turconi et al., 2014). Therefore finding more efficient and sustainable solutions to ensure the provision for the needs of future generations is crucial.

1.2 Biomasses

Finding alternative, clean, sustainable and efficient sources to produce energy, materials, as well as food represents a worldwide challenge.

To meet the sustainability principles these resources should be renewable. A renewable feedstock is a resource that is limitless in supply or that can be replenished over time by natural processes. Therefore, resources such as oil, coal or natural gas, coming from carbon dioxide fixed through photosynthesis millions of years ago, are of limited supplied and cannot be considered renewable. On the contrary wind, solar radiation, tides and biomass can be considered as renewable resources. In particular, a biomass, defined as any organic matter deriving from the remaining of organisms, is particularly

interesting because it can be used not only to produce energy but also chemicals and materials.

Renewable biomasses can be classified into four main categories (Cherubini, 2010) (Figure 2):

1. Agricultural (dedicated crops or residues);
2. Forestry;
3. Domestic- (municipal solid waste and wastewaters) and industrial organic residues (process residues and leftovers);
4. Aquatic (algae and seaweeds);

Figure 2: Biomasses classified in four main categories (Tilman et al., 2009).

Biomasses such as corn, sugarcane and soy, or waste materials, such as forestry, agricultural and industrial wastes, have already been used to produce biofuels or biochemical (Kim and Dale, 2004; Sarkar et al., 2011). However, most of these resources require water and utilize land to grow. This may lead to deforestation and compete directly with food production. Aquatic biomass comprises a diverse group of organisms ranging from macroalgae (multicellular) to microalgae (unicellular) (Bharathiraja et al., 2015). Currently, algae are receiving increasing interest as source of high added value products, such as biochemicals or biopharmaceutics (Alvarado-Morales

et al., 2015; Chew et al., 2017) and biofuels such as biogas or bioethanol (Hou et al., 2015).

1.3 Macroalgae

Macroalgae are a multicellular and macroscopic group of marine algae that live in the sea. This group includes members of red (about 7000 species), brown (about 2000 species) or green (about 1500 species) algae with different biochemical characteristics (Figure 3).

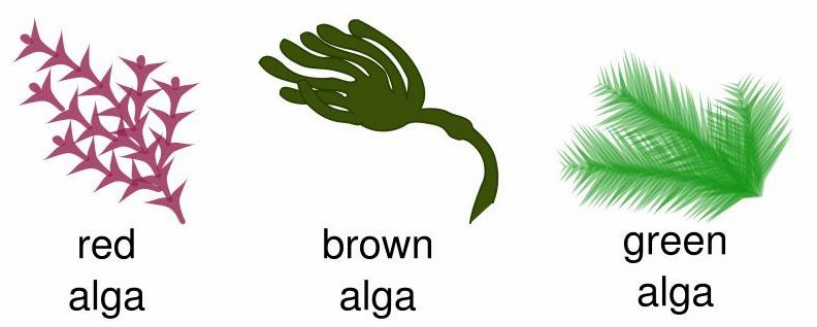

Figure 3: Macroalgae species

They are an interesting source of biologically active compounds such as minerals, vitamins, and proteins that could be used as ingredients in food and feed industries (Evans and Critchley, 2014). Moreover macroalgae have a low caloric value and high amount of dietary fibers whose consumption prevents the occurrence of diseases such as diabetes, obesity, hart diseases and cancer (Kumar et al., 2008; Murata et al., 2002). Furthermore they have antibacterial and anticoagulant activities, and the high polysaccharide content (up to 60% DM) makes them an excellent source for the production of alternative energy or chemicals (Jung et al., 2013; Kraan, 2013). Nowadays biofuels such as biodiesel and bioethanol are mainly produced from terrestrial plants. However the land consumption and the competition with food, characteristics of the first and second generation biomasses, make it necessary to find new feedstocks that comply with sustainability principles. Macroalgae, growing in the sea, do not require agricultural land, freshwater

or fertilizers, thereby overcoming the competition with land-based crops for food and feed production.

Therefore macroalgae can be seen an unexploited and promising resource for the production of third generation fuels, energy, chemicals and materials that can meet the global demand (Enquist-Newman et al., 2013).

1.3.1 Laminaria sensu lato

The most common macroalgae species cultivated in Danish waters are the brown algae belonging to the genus Laminaria sensu lato, *L. digitata* and *S. latissima*. They have been selected because of their high bioremediation potential, yield, growth rate and commercial demand (Handå et al., 2013; Reid et al., 2013; Sanderson et al., 2012). The cultivation of these brown algae has solid documentation in open water rope cultivation (Andersen, 2005), with yields up to 60 t DM ha^{-1} (Bruton et al., 2009).

Nutritional elements such as proteins, lipids, carbohydrates, minerals and vitamins are the main compounds in *L. digitata* and *S. Latissima* biomass. Nowadays *L. digitata* and *S. Latissima* are mainly used as raw material for human consumption or in the phycocolloid industry (Evans and Critchley, 2014). However, recently the interest in using them as feedstock for the production of biofuels and feed to aquaculture has rapidly increased. Macroalgae composition is particularly interesting because, besides their high protein and carbohydrate content, they do not contain lignin (Holdt and Kraan, 2011). The absence of lignin means that pre-treatments before enzymatic hydrolyses of carbohydrates are not necessary, making the biomass processing easier (Enquist-Newman et al., 2013; Wargacki et al., 2012).

Moreover, despite the low content in brown seaweed, the polyphenols represent the most important metabolites in marine algae. They have the potential for reducing the progression of various diseases such as cancer, inflammations, atherosclerosis, cardiovascular disorders and diabetes

(Cardona et al., 2013; Lee and Jeon, 2013; Mayer et al., 2013). For this reason they determine the nutraceutical and pharmaceutical value of algae. Therefore, in a biorefinery perspective, a study to quantify the content of these molecules in *L. digitata* and *S. latissima* species is essential.

However, previous studies demonstrated that the composition of macroalgae varies markedly according to season and geographic distribution (Fleurence, 1999; Ito and Hori, 1989; Schiener et al., 2014). In fact, environmental conditions such as temperature, light, nutrient availability, salinity and water current are key factors in the accumulation of biocompounds in the biomass tissue (Møller et al., 2016). Therefore, the absence of systematic data on seasonal and geographical variations of the biomass composition for these species harvested in Danish waters, makes the evaluation of the biorefinery potential of these macroalgae year-around difficult.

1.4 Microalgae

Microalgae are photosynthetic unicellular organisms found in sediments as well as in marine and freshwater environments. Microalgae comprise a heterogeneous group of species that includes prokaryotic and eukaryotic organisms with the common feature of being able to transform light and carbon dioxide into biomass through photosynthesis. The main nutrients are nitrogen and phosphorous, which have to be bioavailable, e.g. as NH_4^+ and PO_4^{3-}, and different micronutrients like trace metals, vitamins, and others. Algal growth on NH_4^+ as nitrogen source can be simplified by eq. (1):

$$16NH_4^+ + 92CO_2 + 92H_2O + 14HCO_3 + HPO_4 = C_{106}H_{263}O_{110}N_{16}P + 106O_2 \quad (1)$$

Because of their high biochemical diversity and ability to produce different kinds of metabolic compounds, many research efforts in biotechnology are focused on microalgae. The interesting composition in terms of antioxidants, proteins, vitamins and lipids makes microalgae a promising source of metabolites or bioenergy (Chew et al., 2017). Indeed, they are mainly used as

feed or food additives, ingredients in cosmetics or pharmaceutical products or as substrate to produce biofuels such as biogas or biodiesel (Figure 4).

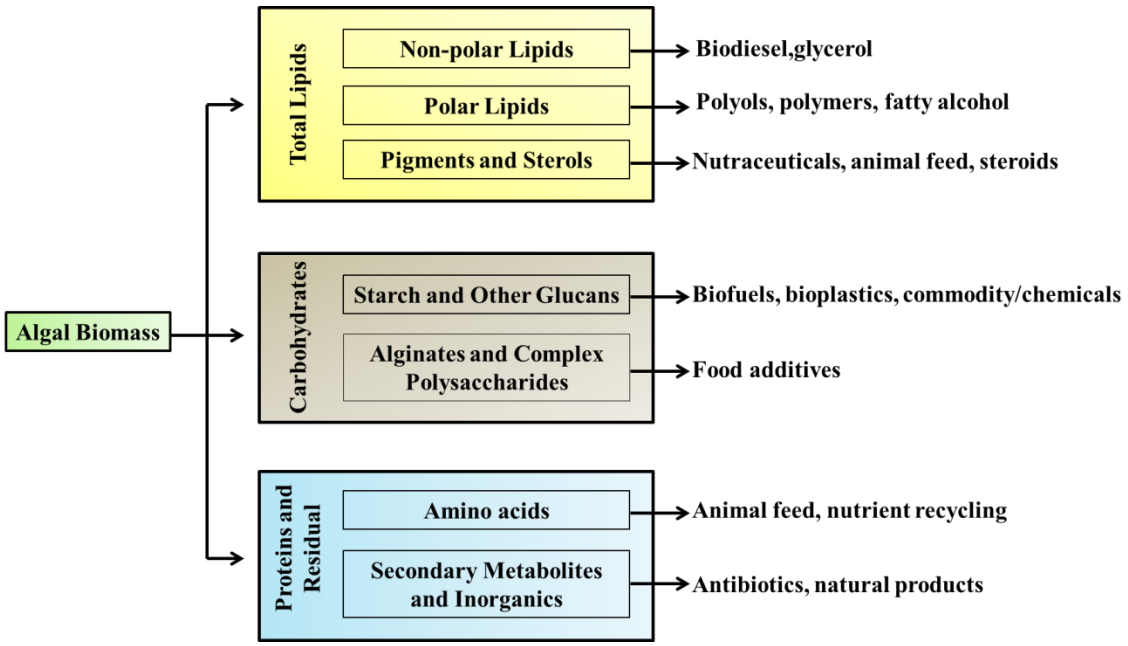

Figure 4: Overview of products from microalgal biomass

One of the most important antioxidants presented in microalgae is lutein. It is a yellow xanthophyll, member of the carotenoid group, that plays a fundamental role in preventing some types of cancer and diseases in both humans and animals (Astorg, 1997; Chiu and Taylor, 2007; Dwyer et al., 2001; Granado et al., 2003). Despite the high content of lutein in microalgae, nowadays the main source for extraction and production of this pigment are marigold flowers. The main limitation in the exploitation of algal biomass for lutein production is related to the low yield obtained in the extraction process. Therefore the development of an optimized protocol to extract lutein from microalgae constitutes a remarkable feature with the potential to increase the economic indicators of a biorefinery facility. Moreover, microalgae represent an interesting resource because they can be cultivated in areas not suitable for plants, they do not suffer from any seasonality effect and their production rate is extremely high.

Furthermore, microalgae have another interesting potential application. Since they are eukaryotic organisms, they can synthesize proteins with proper modifications to be functional in humans. Indeed, because of innovative genetic engineering tools and the availability of algal genome data, microalgae have been engineered and used as cell factories. Moreover, microalgae cultivation is more cost effective than developing mammalian cell cultures, since they are easy to grow and maintain (Pei et al., 2010).

The most commercially interesting microalgae belong to *Chlorella*, *Spirulina*, *Dunaliella* and *Haematococcus* genera (Bruton et al., 2009). They are able to grow in different environments such as freshwater, marine water and soil (Pei et al., 2010). Some of the species that belong to this genus are *Chlorella vulgaris*, *Chlorella kessleri*, *Chlorella lobophora* and *Chlorella sorokiniana*.

1.4.1 Chlorella

In particular Chlorella is a genus of unicellular green algae. A brief overview of the most important species is reported below.

- ***Chlorella sorokiniana***:

  Its cells size ranges from 2 to 15 μm in diameter with a round or ellipsoidal shape (Figure 5).

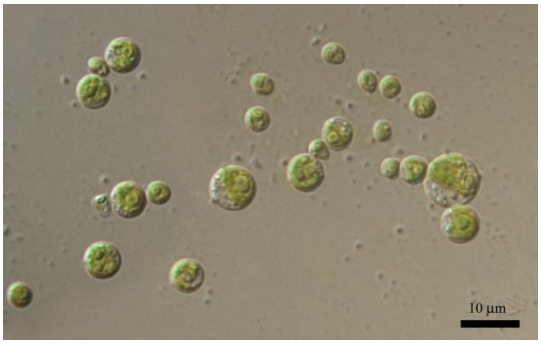

Figure 5: *C. sorokiniana* cells

It grows optimally at 37-38°C, pH 6.5-7.0. Previous studies showed that proteins, carbohydrate, lipids and total carotenoids in *C. sorokiniana* comprised 68.5, 11.9, 10 and 0.69% of DM, respectively

(Matsukawa et al., 2000). The microalga is able to withstand high temperatures and light intensities producing protection agents such as antioxidants and pigments. The carotenoids potentially interesting for commercial use in a biorefinery perspective found in *C. sorokiniana* are alpha and beta-carotenes, lutein, zeaxanthin, violaxanthin and neoxanthin. Moreover it contains all the essential amino acids, enabling the production of single cell proteins used as supplement in human and animal nutrition.

- ***Chlorella vulgaris:***

  It has spherical cells with 2-10 µm in diameter (Safi et al., 2014) (Figure 6).

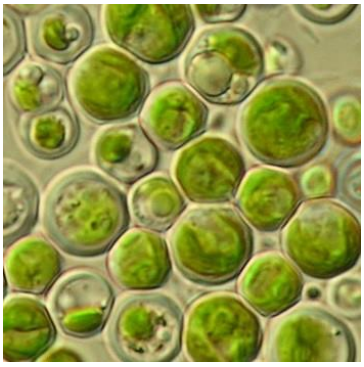

Figure 6: *C. vulgaris* cells

It grows optimally at 30°C, pH 6.5-7.0. The total proteins, lipids and pigments in *C. vulgaris* biomass can reach 42-58%, 5-40% and 1-2% of dry weight, respectively (Safi et al., 2014). Lutein is one of the main carotenoids in *C. vulgaris.*

- ***Chlorella protothecoides:***

  Its cells are small and round (Figure 7).

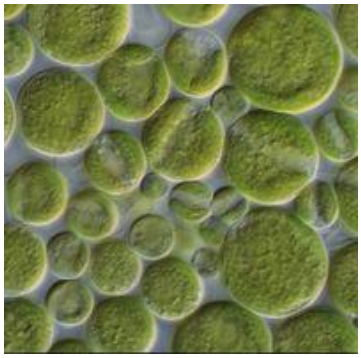

Figure 7: *C. protothecoides* cells

Its optimal temperature is 28°C, while pH is 6.5-7.0. Previous studies reported a microalga proteins composition on a dry weight basis up to 50% (Xu et al., 2006a).

1.4.2 Microalgal culture conditions

Microalgae are able to adapt their metabolism to changing environments. Different culture conditions may influence the algal productivity and final composition of a microalga. Therefore an analysis of the effect of different parameters on the microalgae composition is crucial. Light in particular represents one of the most important parameters. Most of the microalgae metabolites are obligate photoauthotrophs, therefore they require light (natural or artificial) to grow (Lee, 2001). However some species are able to utilize the carbon sources in the medium to gain energy for their metabolism, according to a heterotrophic configuration.

1.4.2.1 Phototropic cultivation

The most common configurations to grow microalgae photothrophically can be seen in Figure 8 and can be divided into three groups:

1. Natural habitats (lakes, lagoons and ponds);
2. Open pond reactors;
3. Closed photobioreactors (PBRs).

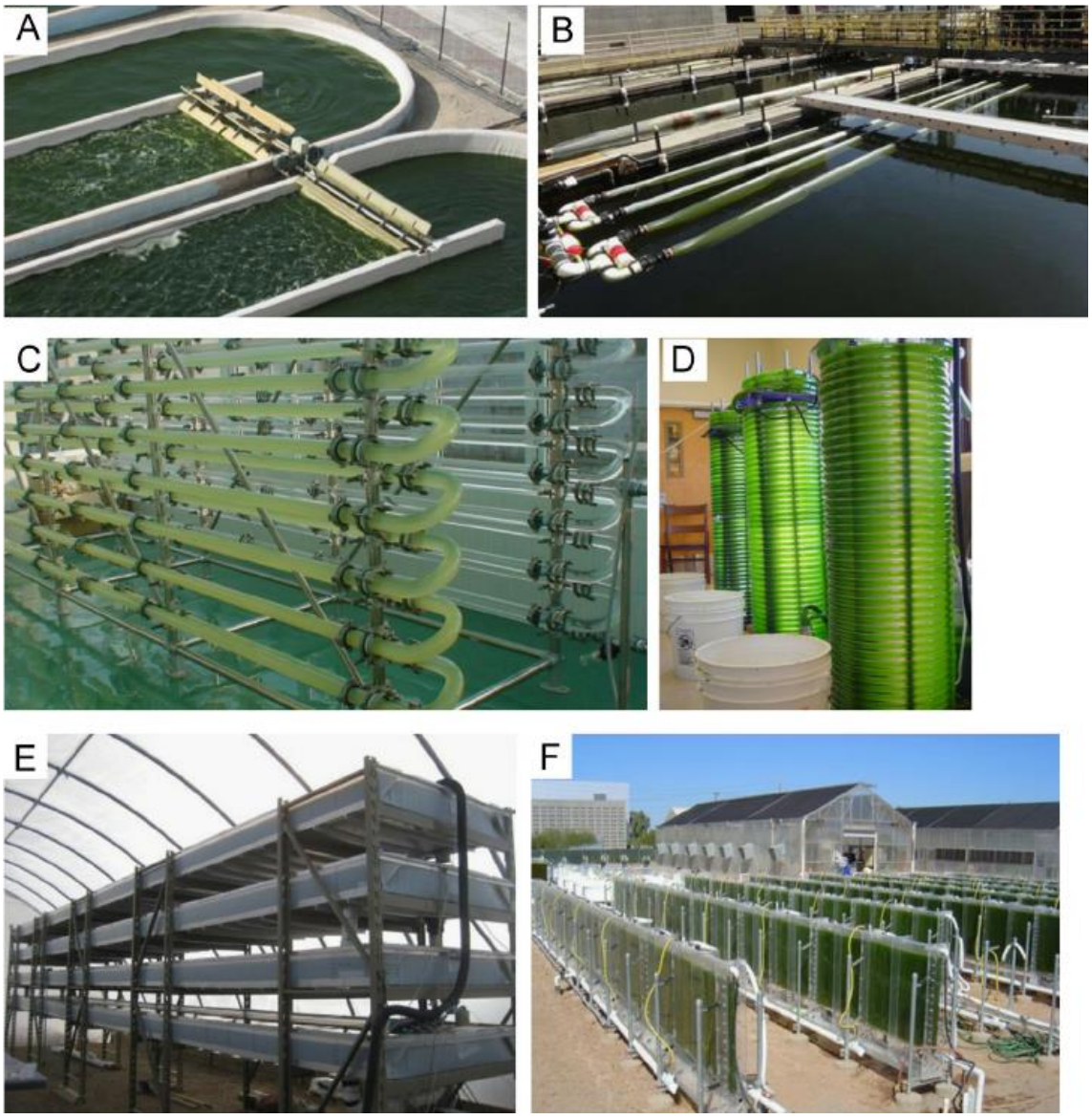

Figure 8: Different reactors for large-scale microalgal cultivation: (A) raceway pond; (B) floating photobioreactor; (C) tubular bioreactor; (D) coil bioreactor; (E) multi-layer bioreactor; (F) flat-panel bioreactor (Zhou et al., 2014).

The main configuration for large scale microalgae cultivation is represented by the open pond reactors under sun light. This configuration is extremely simple and the CO_2 necessary for microalgae growth is taken directly from the surface air. However, the huge volume of these reactors leads to difficult and costly harvesting processes. Moreover, the contamination risk is extremely high and therefore obtaining mono-cultures is difficult. For this reason the species need to have high growth rates or to be adapted to grow in a specific growth medium that prevents contamination. Moreover, when algae

are grown in regions with warm temperatures and high light intensities, the water evaporation in open reactors is huge. Furthermore, when cell densities increases, the light penetration decreases. As a consequence, algae far from the top of the reactor do not have sufficient light and the growth and productivity decrease.

Therefore, to have a better process control overcoming the weaknesses of the open pond system, PBRs are used. These reactors can ensure stable and selective environments, controlling parameters such as light intensity, pO_2, pCO_2, temperature, pH, mixing and substrate addition for microalgae growth (Zittelli et al., 2013).

However, the main drawbacks of PBRs are the high capital and operating costs, the energy consumption and the limited possibility of being scaled up (Lehr and Posten, 2009). Intensive research to develop simple, low-cost and easily scalable PBR design has been done (Brennan and Owende, 2010; Morweiser et al., 2010; Posten, 2009; Zittelli et al., 2013). Different configurations, such as flat or tubular, horizontal, inclined, vertical or spiral, manifold or serpentine, hybrid, floating or biofilm reactors have been analyzed (Figure 8). Moreover, to maximize productivity and yield and simplify operation and handling of the microalgae, intensive mixing (Richmond, 2004), light dilution applying various techniques (Jan-Willem F Zijffers et al., 2008; Jan-Willem F. Zijffers et al., 2008), and cultivation of genetically improved strains were applied (Radakovits et al., 2010). However, the costs for the system sterilization to prevent contaminations and for scaling up and operate the reactors are high. Moreover, the presence of a constant intense illumination, fundamental to achieve a high productivity, increases the costs associated to the process (Richardson et al., 2014).

Therefore, an optimization of the existing technologies together with the development of novel configurations not depending on the light supply is crucial to increase the industrial competitiveness of the microalgae processes.

1.4.2.2 Heterotrophic cultivation

Some microalgae species are able to grow in the dark, utilizing an organic carbon substrate rather than CO_2 and light to supply energy to the cells growth. The advantages associated to the heterotrophic microalgae cultivation are: elimination of light requirements, easier operation of bioreactors and increased growth rates and productivity of proteins and lipids. The organic carbon sources that these algae are able to metabolize are pyruvate, acetate, lactate, ethanol, saturated fatty acids, glycerol, C6 sugars, C5 monosaccharides, disaccharides and amino acids (Morales-Sanchez et al., 2014). However the cost of these carbon sources represents the main drawback of this operation mode. Previous studies demonstrate that about 80% of the total medium cost is represented by the cost of the glucose (Li et al., 2007). Therefore the need of finding alternative low cost carbon sources is impellent. Indeed, recent studies focused on the utilization of inexpensive carbon sources such as food waste hydrolyzed or whey permeate as nutrients source in microalgae cultivation (Espinosa-gonzalez et al., 2014; Pleissner et al., 2013). However, more research on cheap carbon sources is needed for the development of cost-efficient processes for food, feed, and biofuels production.

1.5 Biorefinery

The conversion of biomass into chemicals, biomaterials and energy with the aim of maximizing the value of the raw material and minimizing the wastes, making the overall process economically feasible is obtained by means of a biorefinery (González-Delgado and Kafarov, 2011)

According to the IEA Bioenergy Task 42 on Biorefineries, a biorefinery is defined as "*the sustainable processing of biomass into a spectrum of marketable products and energy*" (de Jong et al., 2009) (Figure 9).

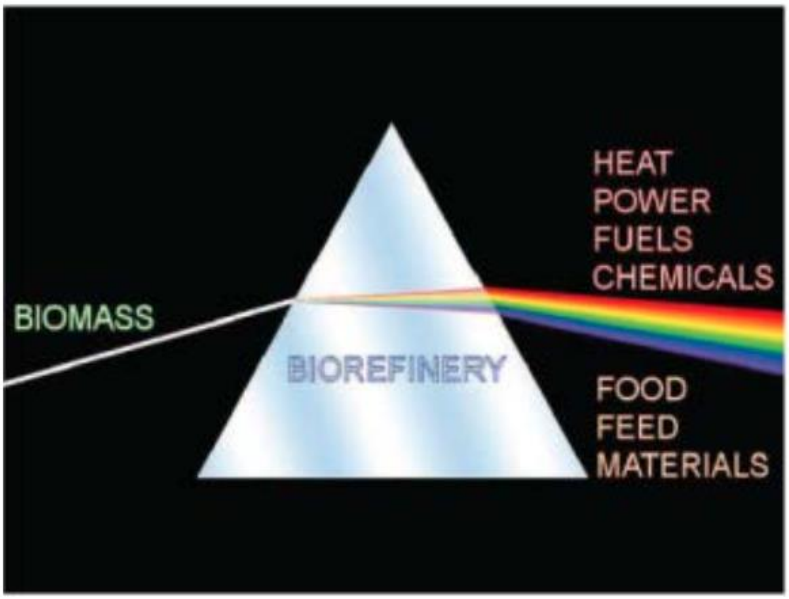

Figure 9: Schematic overview of a biorefinery

The biorefinery concept is similar to the oil-based refineries, where at least two marketable chemical products plus at least one energy form are produced from oil. Therefore a biorefinery employs biomass instead of non-renewable resources to obtain different outputs (Figure 10).

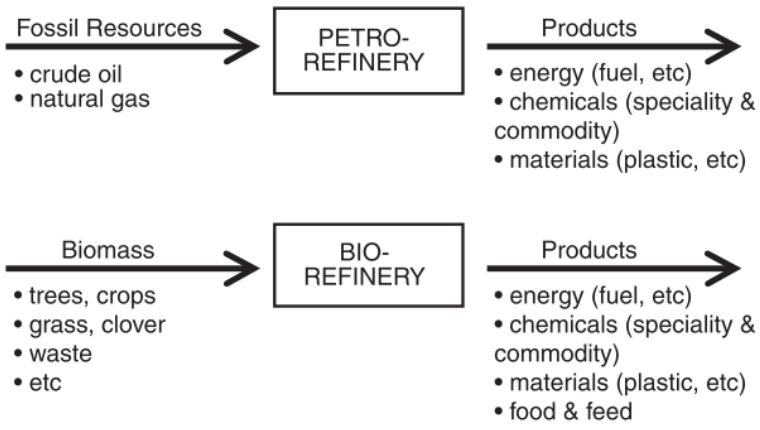

Figure 10: Comparison between a petrorefinery and a biorefinery (Clark and Deswarte, 2008).

In a biorefinery low value, high volume products such as biodiesel or bioethanol, and high value, low volume products such as cosmetics, pharmaceuticals or nutraceuticals are produced from biomass (Figure 11) (Clark and Deswarte, 2008).

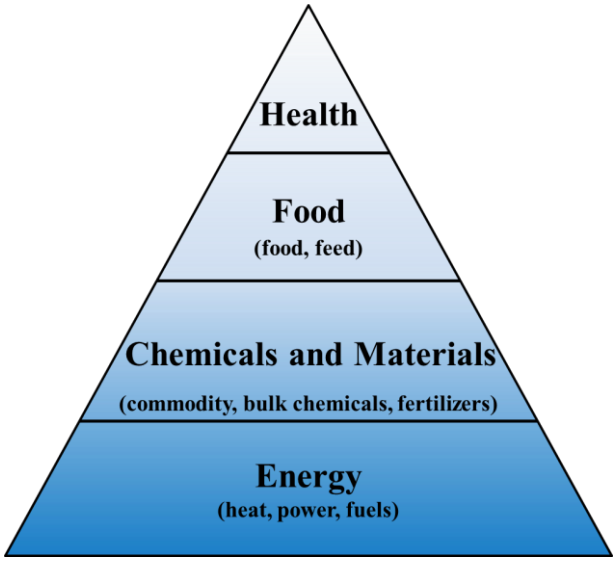

Figure 11: Value pyramid of renewable feedstock

Among all the compounds in the biomass four constituents are the most relevant in the production of biofuels and bioproducts: carbohydrates, lignin, triglycerides and proteins (Cherubini, 2010).

- **Carbohydrates,** such as glucose, galactose, mannose or xylose and arabinose are the most common component in plant biomasses. Sugar crops such as sugar cane and sugar beet, together with corn, represent the main source of bioethanol (Cherubini, 2010).
- **Lignin**, the largest non-carbohydrates component of the lignocellulosic biomass, cannot be used in fermentation processes but it is used in chemical extractions or energy generation.
- **Triglycerides**, glycerine and saturated and unsaturated fatty acids, are mainly present in vegetable and animal raw materials. Vegetable oils, such as soybean, palm, rapeseed and sunflower, represent the main source of biodiesel. However, other sources of vegetable oils can be the waste streams from food processing plants or commercial services (Cherubini, 2010).
- **Proteins** are mainly contained in seeds of legumes, oilseed meals, soya and wheat. The extraction of high quality proteins without affecting their chemical properties is difficult and new improved methods are subject of

current research (Clark and Deswarte, 2008). The applications of these vegetable proteins are mainly in the food and cosmetic sector. However, specific proteins to be used for the healthcare sector, such as antibodies, antigens and vaccines, have been developed using transgene technologies (Chen and Davis, 2016; Yao et al., 2015). The production of plant derived pharmaceuticals due to the low production cost, the high scalability and the safety of the process, has attracted great interest (Chen and Davis, 2016). Among the several approaches developed, the use of algal bioreactors represents a promising strategy in the production of vaccines, enzymes and antibodies (Yao et al., 2015).

Therefore knowing the composition of the specific feedstock is crucial in determining the specific biofuels and the biochemicals that can be produced in a biorefinery.

According to literature, biorefineries can be classified based on their production technologies in (Clark and Deswarte, 2015; Fernando et al., 2006; Kamm and Kamm, 2004):

- **First generation biorefineries**: use of classical agricultural biomasses such as rapeseed, sunflower or sugar cane. They normally use sugars, starch, vegetable oils or animal fats to produce biofuels using conventional technologies. In Europe there are many companies producing biodiesel according to this configuration. However risk of deforestation, excessive utilization of fertilizers and competition with food crops are the main issues related to the first generation biorefineries (Clark and Deswarte, 2015; Gomez et al., 2008).

- **Second generation biorefineries**: use of lignocellulosic biomass, such as straw, plant trunks and wood as feedstock to produce energy and value added products. Therefore the reduced dependence on food crops and the diversity of products that can be obtained with these biomasses

allows the penetration into new markets generating substantial revenues. However the high cost of enzymes necessary to break down the lignocellulosic materials is one of the main issues associated to the second generation biorefineries.

- **Third generation biorefineries:** use of agricultural and forestry residues, urban wastes and algae. They are the most advanced biorefineries due to their capability to produce a variety of products using a wide range of different feedstocks. Besides the advantage of high market adaptability, the utilization of multiple feedstocks secures the possibility of selecting the most profitable combination of raw materials. The first demonstration-scale example of third generation biorefinery in the world is based in Oulu, Finland, on the Chempolis' Formico platform (Demirbas, 2010a). The plant processes wood or non-wood or non-food raw materials such as straw, grass, bagasse and leaf fiber into bioethanol, biochemicals, and papermaking fibres.

  However, more research is needed to improve the technological processes and the biomass conversion efficiency of the third generation biorefineries. The interest in using new biomasses, such as algae (macro- and micro-) has recently increased (Chew et al., 2017; Jung et al., 2013). Algae have a high potential, since they are able to generate more biomass than terrestrial crops do and, due to their high photosynthetic ability, they are able to produce and store sufficient resources necessary for biorefinery processes. Therefore, algae may become a key element in the future chemical and biochemical industries, in a wide range of sectors such as food, feed and pharmaceuticals (Vanthoor-Koopmans et al., 2013).

1.6 Objectives and thesis structure

Based on challenges described, the main objectives of this PhD project were to investigate innovative concepts to produce proteins and other high value products from macro and micro algae. Additionally, a preliminary study to evaluate the effect of the harvesting time and of the geographical location on composition of the macroalgae *L. digitata* and *S. latissima* was done. This biomass characterization was performed in order to test their potential as feedstock for biorefinery.

In order to fulfil the main objective, the following specific objectives were:

- Determine seasonal and geographical variations in the biogas yield and total phenolic compound content in macroalgae *L. digitata* and *S. latissima* for evaluating their biorefinery potential.
- Analyze different industrial methods to produce proteins and amino acids, underlining the importance of the fermentation process and the potential of innovative approaches utilizing single cell proteins (SCP).
- Test the potential of *L. digitata* as feedstock for heterotrophic growth of three different microalgae species, namely *C. protothecoides*, *C. vulgaris* and *C. sorokiniana,* identified as a valuable source of proteins.
- Develop an innovative biorefinery process where the autotrophic growth of the microalga *C. sorokiniana* in biogas is combined with the production of SCP utilizing *M. capsulatus.*
- Develop a novel method capable to extract lutein from microalgae with a high purity and recovery.
- Implement a microalga biorefinery concept where lutein, proteins and biogas are produced.

In Chapter 2 results on the effect of the cultivation site and season of harvest on macroalgae *L. digitata* and *S. latissima* composition are given (Paper I).

In Chapter 3 the different approaches develop in this work to produce proteins are discussed (Paper II). Moreover case studies of potential biorefinery schemes to produce SCP utilizing *M. capsulatus* (Scientific Report I) and combining macro and microalgae are presented (Paper III).

In Chapter 4 the potential of microalgae biorefinery is evaluated and the results of the novel method to extract lutein (Paper IV) and proteins from microalgal biomass are presented (Paper V).

2 Macroalgae: seasonal and geographical effect

Brown macroalgae, such as *L. digitata* and *S. latissima*, are a promising candidate to be used in future biorefineries concepts to produce third generation biofuels and materials (Enquist-Newman et al., 2013; Jung et al., 2013). However, previous studies demonstrated that overall chemical composition of macroalgae varies markedly according to season and geographic distribution (Fleurence, 1999; Ito and Hori, 1989; Schiener et al., 2014). Systematic analyses of the effect of harvesting season and geographical location on the biomass composition are crucial to evaluate the biorefinery potential of these macroalgae year-around. In this chapter results respect to the effect of the seasonality and geographical location on the total phenolics composition and on the biogas potential of *L. digitata* and *S. latissima* harvested throughout a year and in different locations in Denmark are presented. The total phenolics were determined as they may represent an interesting bioactive compound, due to their health benefits. Although the biogas and total phenolics variation of brown algae has been reported before (Adams et al., 2011; Schiener et al., 2014), no information is available on the seasonal and geographical variation of these species in Danish waters.

2.1 Seasonality effect

In Paper I the seasonality effect on the total phenolics and the biogas potential of *L. digitata* and *S. latissima* harvested from wild stocks throughout 2013 from Århus Bugt and Hanstholm, Denmark, was assessed.

This study proved that the seasonality significantly affects the biogas production of the macroalgae harvested in Danish waters. The biogas potential varied from a minimum of 155.3 and 213.8 $NmLCH_4$ g VS^{-1} in summer to a maximum of 358.9 and 285.0 $NmLCH_4$ g VS^{-1} in winter for *L. digitata* and *S. latissima* samples respectively (Figure 12).

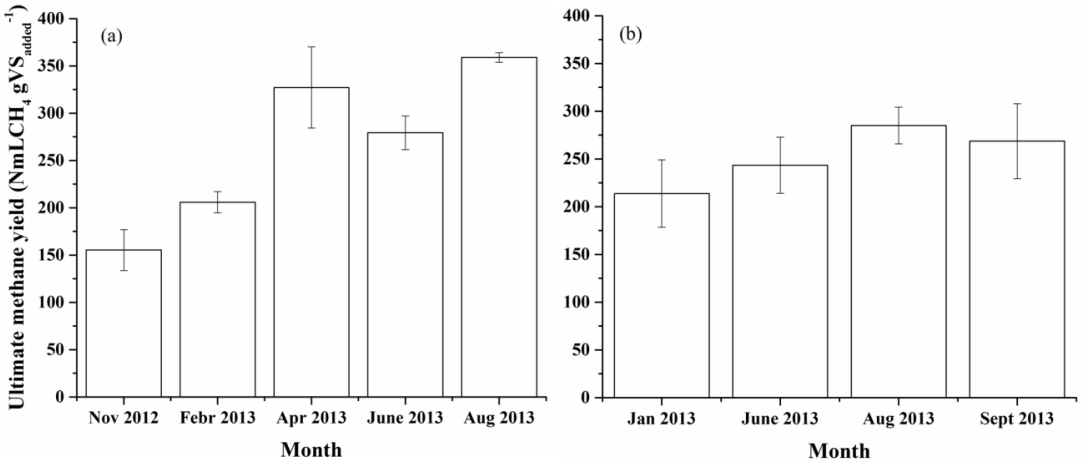

Figure 12: Ultimate methane potential for (a) *L. digitata* and (b) *S. latissima* harvested in Århus Bugt after 30 days incubation, error bars show standard error.

To explain this behavior, an evaluation based on previous studies on these two macroalgae species, of proteins and carbohydrates, the main biogas sources in an anaerobic digestion process, was done. Manns et al. (2017) demonstrated that the total sugars content reaches its maximum during summer. Conversely proteins content varies from 3 to 21% g gDM^{-1} being much higher in winter than in summer for both macroalgae (Yang et al., 2015). Despite the high protein content of the samples analyzed in this study (around 15% g gDM^{-1} in winter and 8% in summer) for both the macroalgae (Manns et al., 2017), previous studies demonstrated that proteins have lower biodegradability than carbohydrates (Yang et al., 2015). Therefore a direct consequence of this behavior is the higher biogas produced during summer. Moreover the C:N ratio of all the samples was determined. The anaerobic digestion (AD) is a complex process that involves different consortia of microorganisms that in various steps are able to convert a biomass into biogas. An imbalance in the process due to a change of temperature, pH or to the presence of inhibitors such as organic acids is critical for the microbes activity with a consequent lower biogas production. In particular the most common inhibitor in an AD process is represented by the ammonia (Chen et al., 2008). Therefore the evaluation of the C:N ratio must be considered as a

preliminary criterion to assess a specific substrate for biogas production. A nitrogen rich substrate, represented by a C:N ratio below 20:1, indicates that there is an imbalance between carbon and nitrogen requirements for the growth of the anaerobic microorganisms. This can result in an increased level of ammonia in the reactor which can eventually lead to process inhibition (Allen et al., 2013).

The results are confirmed by the C:N ratio (Figure 2 in Paper I). Indeed for an optimal performance of an anaerobic digestion process the C:N ratio should be in the range 20:1–30:1. This optimal ratio was observed in August for *L. digitata* and for *S. latissima*.

Moreover the seasonality effect on the biogas potential was tested also of *L. digitata* harvested from wild stocks throughout 2013 from Hanstholm, Denmark (Figure 13).

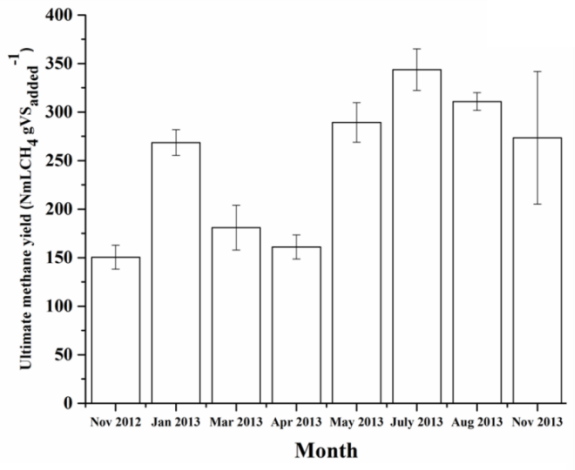

Figure 13: Ultimate methane potential for *L. digitata* harvested in Hanstholm after 30 days incubation, error bars show standard error.

Samples harvested in July produced the highest cumulative volume of methane 343.7 $NmLCH_4$ g VS^{-1}. The total sugar content in summer in these samples is very high (glucose content of 54.0% g gDM^{-1}), while the proteins remained at a low range (around 6.0% g gDM^{-1}) (Manns et al., 2017). The

same considerations done for *L. digitata* harvested in Århus Bugt can be done for the specie harvested in Hanstholm.

Furthermore, comparing the biogas produced from samples harvested in the North Sea with the ones from the Danish Baltic Sea a different trend was observed. Therefore, the environmental conditions have an effect on the biomethane production. Water motion and temperature, light, nutrients concentration, flow rate, depth, pH may influence the macroalgae composition.

Hanstholm was identified as a better location to produce biogas, therefore the seasonality effect on the TPC content in *L. digitata* was analyzed in this harvesting site.

The highest content of TPC was recorded in summer (0.5 mgTPC gDM^{-1}) and the lowest levels in autumn (0.1 mgTPC gDM^{-1}) (Figure 14).

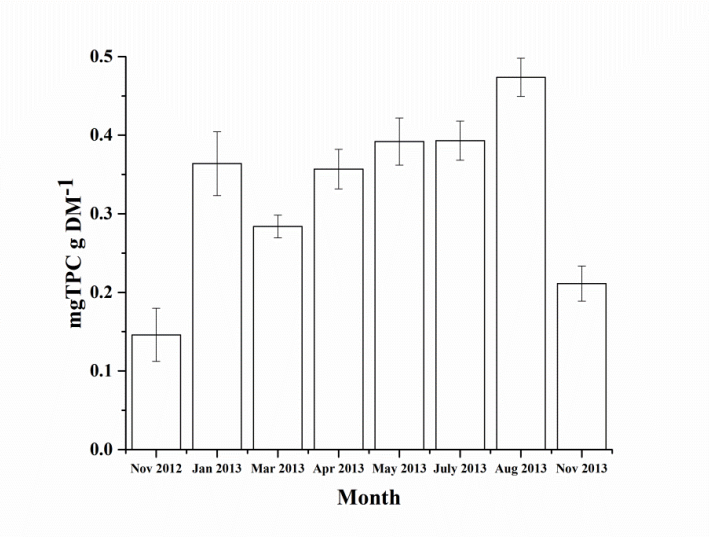

Figure 14: Total phenolics for *L. digitata* harvested in Hanstholm expressed as mgTPC 100g DM^{-1}, error bars show standard error.

A different light intensity among the seasons is the main factor responsible of the TPC seasonal differences. In summer, when the irradiation is high, high reactive oxygen molecules are produced. These molecules may destroy the algae photosynthetic system leading to the cell death. Therefore algae

developed photoprotective mechanism to protect themselves. Phenolic compounds act as a buffer deactivating the highly reactive form of oxygen. Hence during summer their content increases with a consequent increasing in the antioxidant activity.

The results proved that summer is the best season to harvest macroalgae to produce biogas and TPC.

2.2 Geographical effect

In Paper I *L. digitata* and *S. latissima* samples harvested in August 2012 respectively from 12 and 8 different locations in Denmark were analyzed (Figure 15).

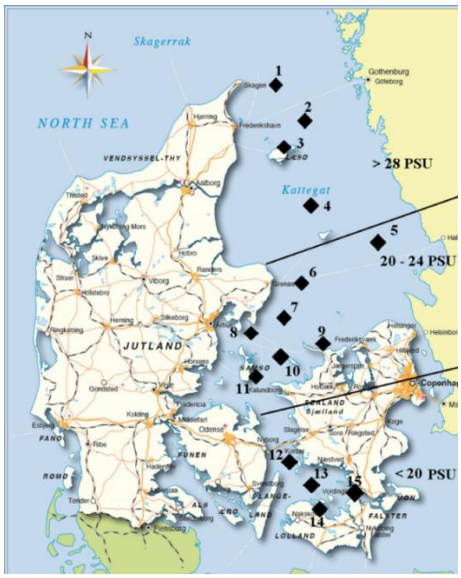

Figure 15: Map with sampling sites for L. digitata and S. latissima and salinity (PSU)

A fluctuation of salinity is observed in the Kattegat and in the Skagerrak. In the northern Kattegat the level of salt in the water is high (up to 30 PSU) and it gradually decreases to 20 PSU in the southern part.

The highest methane yield was achieved in site 4 (393.8 $NmLCH_4\,gVS^{-1}$) for *L. digitata* and 8 (383.3 $NmLCH_4\,gVS^{-1}$) for *S. latissima* (Figure 16).

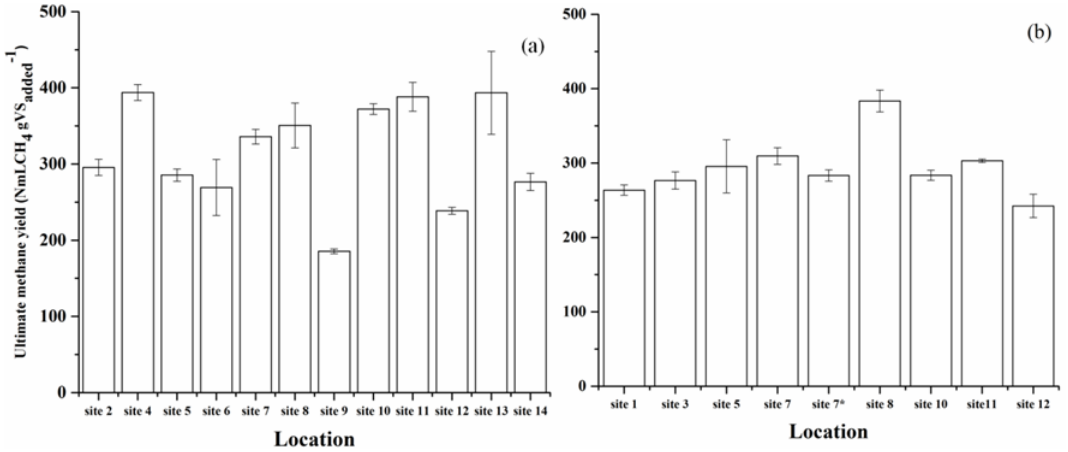

Figure 16: Ultimate methane potential for *L. digitata* (a) and *S. latissima* (b) harvested in different locations in Denmark after 30 days incubation, error bars show standard error.

Møller et al. (2016) demonstrated that the sugars, as well as the protein content, varied strongly among the sites. In this work a high level of sugars was recorded in the samples harvested in station 9 for *L. digitata* and station 8 for *S. latissima*. According to their study, that station 8 represents the best location to harvest *S. latissima* for biogas production. However site 9 is not the best harvesting location for *L. digitata*. This is due to a lower biodegradability of the substrate harvested in site 9 (46.2%) than the other sampling sites.

The impact of the harvesting site on TPC accumulation was also investigated.

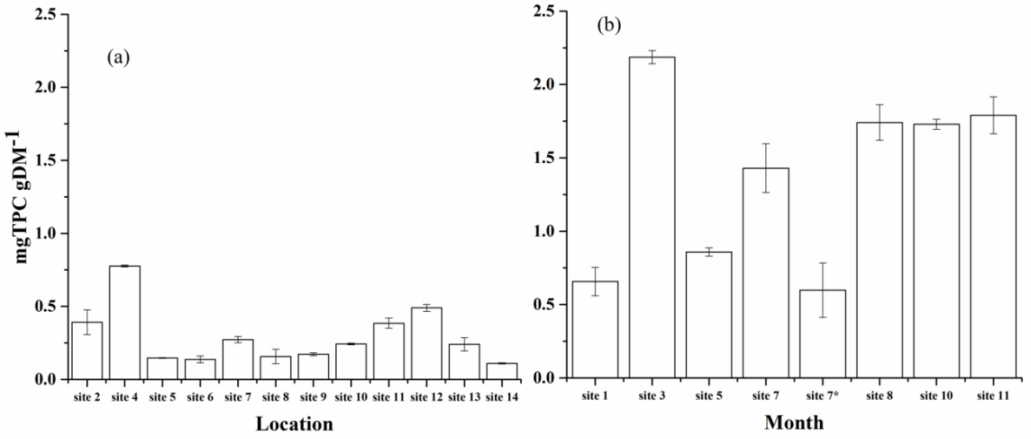

Figure 17: Total phenolics for *L. digitata* (a) and *S. latissima* (b) harvested in different locations expressed as mgTPC 100g DM^{-1}, error bars show standard error.

Firstly was observed that the TPC content in *S. latissima* is much higher than in *L. digitata*. Secondly the highest content of phenolics compound was recorded in site 4 for *L. digitata* (0.8 mgTPC gDM^{-1}) and 3 for *S. latissima* (2.2 mgTPC gDM^{-1}). Both the locations are situated in an area of high salinity (>28 PSU). Indeed previous studies demonstrated that a reduction in salinity is associated with a different level of salts in the marine environment influences the ionic concentration, the density, the nutrient uptake and the pH of the water with a consequent decreasing in the TPC content (Connan and Stengel, 2011). However, a strong relationship between salinity and TPC content was not found. A possible explanation is that the macroalgae were harvested at different depths and therefore were subjected at a different irradiance. To confirm this assumption samples from different depths 7m (site 7) and 11m (site 7*) harvested in the same location were analyzed (Figure 9 b). The results show that the higher irradiance at 7m increases the photosynthesis with a consequent higher TPC accumulation.

Therefore the results obtained demonstrated that *L. digitata* and *S. latissima* harvested in Danish waters are affected by seasonal and geographical variation. The best season to produce biogas and TPC is summer when the sugars in the samples and the irradiance reach their maximum. Moreover a direct correlation between salinity and biogas production and TPC accumulation was not found. However Hanstholm, due to the high carbohydrate content in the samples harvested in this location, represents an interesting location to grow algae to be used in a biorefinery prospective.

3 Production of proteins and amino acids

Human food security requires the production of sufficient amounts of proteins and amino acids (Coles et al., 2016). Nowadays, the main source to replace animal proteins in human nutrition are plants (Day, 2013). They play an import role as food additives such as emulsifiers and gelatinous agents (Day, 2013). Moreover, from a nutritional point of view, plant proteins are a source of all essential amino acids. However the content of essential amino acids in plant proteins is usually reduced in comparison to animal proteins, in particular for the amino acids methionine, lysine and tryptophan (Krajcovicova-Kudlackova et al., 2005). It is well documented that amino acids are crucial to promote health. They maximize the efficiency of food utilization, reduce the adiposity, regulate the muscle protein metabolism and control the growth and immunity of the organism (Weinert, 2009; Wu et al., 2004; Yamane et al., 2007). Indeed they are involved in the regulation of key metabolic pathways and processes that are crucial for the growth and the maintenance of organisms (Cesari et al., 2005; Wu, 2009). For these reasons, amino acids and proteins are used in several industrial applications as bulk biochemicals used to produce many products such as animal feed additives, flavour enhancers in human nutrition or as ingredients in cosmetic and medical products. Therefore their market demand is steadily increasing and the interest in developing more cost-effective and sustainable process to produce them is raised.

3.1 Amino acids production processes

Paper II describes how amino acids can be produced. They are produced by different processes such as extraction from protein hydrolyzates, chemical synthesis, enzymatic and fermentation pathways with the aid of microorganisms. In particular the fermentation process, because of the new genetic engineering tools applied to optimize yields, specificity and

productivity of amino acids production, is becoming one of the most promising processes for amino acids commercial production (Ikeda, 2003). In this paper the main advantages and disadvantages of each method are presented (Table 1 in Paper II). Moreover process parameters, technological issues associated to an industrial amino acid plant, possible improvements and the potential of innovative approaches utilizing macro and microalgae or bacteria are also discussed.

3.1.1 The fermentation process

Most of the industrial processes to produce amino acids are based on the fermentation (Ikeda, 2003). The main advantages of this process are the low maintenance costs and the possibility of producing only the L-form amino acids avoiding further purification steps (Ugimoto, 2010). However, fermentation requires sterility and high energy consumption for oxygen transfer (for the aerobic fermentations) and mixing, as well as water addition that impact on capital and operation costs. Moreover it requires a bigger reactor compared to the other production methods, with a consequent higher capital investment (Ivanov et al., 2013). Therefore the production process can still be optimized. For this reason the research, with the aim of producing amino acids in a cost-effective and sustainable way, has increased (Breuer et al., 2004; Kim, 2010; Kumagai, 2013).

The main improvements involve:

- **Genetically modified microorganisms:** The most common bacteria used to produce amino acids are *Corynebacterium glutamicum* and *Escherichia coli* (Ikeda, 2003). New genetic engineering techniques involving point mutations in genes relevant for the target amino acid have been applied to these microorganisms to maximize their performance (Wendisch et al., 2006). Optimized microorganisms have been constructed utilizing techniques such as riboswitch and CRISPRi for pathway engineering, strongly enhancing the amino acids production (Cleto et al., 2016). Therefore a higher yield,

specificity and productivity of amino acids, and a larger range of fermentable carbon sources and the products that can be obtained from this bacterium have been obtained.

- **Downstream and purification:** Among the most efficient techniques developed to increase the processes performance and hence raise the revenues, the combination of nanofiltration membranes with electrophoresis represents an interesting solution (Kattan Readi et al., 2014). Combining the effect of the iso-electric separation with the membrane selectivity an amino acid recovery of 85% was achieved, demonstrating the great potential of these techniques for industrial applications (Kumar et al., 2010). Moreover, these membranes can be easily integrated with the conventional fermentors combining production and purification in the same operation unit. This reduces the capital investment and leads to the possibility of process intensification (Kattan Readi et al., 2014; Kumar et al., 2010).
- **Fermentor scale-up:** Industrial bioprocesses are often affected by lower mixing efficiency with a consequent lower process stability, reproducibility and yield and with the formation of unwanted by-products that may affect the final product quality (Takors, 2012). Therefore, to develop the optimal process configuration in the early stage of the process, a combination of the results obtained by the scale down devices and by the process modeling techniques has been applied (Elmar et al., 2007; Käß et al., 2014; Lemoine et al., 2015). Such approach can help in the optimization of existing processes but above all it represents a useful support to develop new plants.

3.2 Novel approaches to produce proteins

To increase the microbial cell productivity novel approaches by means of a protein up-concentration have recently been developed by Alvarado-Morales et al. (2015). They extracted the carbohydrate fraction from the macroalgae *L. digitata* through enzymatic hydrolysis; then the liquid hydrolysate was separated from the solid leftover by centrifugation and they obtained a

residue rich in proteins (3.5 fold higher than the original substrate) with a higher bioavailability and digestibility. As a result, a final product with a high nutritional value of the protein that can be potentially used as bioactive compound in food, feed or pharmaceuticals was obtained.

A different methodology to produce proteins is represented by the growth of microorganisms suitable for the production of single cell proteins (SCP). SCP can be produced by algae, fungi, yeast and bacteria such as methylotrophs and hydrogen-oxidizing bacteria and a variety of inexpensive substrates and wastes can be used as growing media (Nasseri et al., 2011). In particular, microalgae and methylotropic bacteria represent an interesting source of proteins. Indeed they can reach a protein content of 40-70% of the dry weight (Becker, 2007; Costard et al., 2012; Eckert and Trinh, 2016; Gatenby et al., 2003). Their growing rate is extremely high and a wide range of growing media can be used (Bumbak et al., 2011; Ramos Tercero et al., 2014; Xu et al., 2006b).

3.2.1 Heterotrophic growth of protein rich microalgae

Paper III demonstrated that an integration of micro- and macroalgae to obtain a final product rich in proteins seems to be an attractive approach. Indeed microalgae are able to grow at different environmental conditions, adapting their metabolism with a consequent change in the final biomass composition. This characteristic allows to tailor the processes in order to maximize the formation of target compounds without any genetic modification.

Mann et al. demonstrated that the macroalgae *L. digitata* harvested in August in Hanstholm (Denmark) can reach carbohydrate content up to 51% of the dry weight (Manns et al., 2014). This suggests that *L. digitata* is a suitable substrate for microalgae heterotrophic cultivation.

In Paper III the use of *L. digitata* as substrate to grow heterotrophically microalgae species rich in proteins to be used as fish feed supplement was investigated.

The macroalgae hydrolyzed used in this study was obtained through enzymatic hydrolysis. This step enables the release in the liquid phase of the sugars in *L. digitata*. A consequent pre-screening experiment to test the ability to grow heterotrophically in the macroalgae hydrolyzate three different microalgae species, namely *C. protothecoides, C. vulgaris* and *C. sorokiniana,* identified as valuable sources of proteins, was done. *C. protothecoides,* because of the shorter lag phase observed, was chosen to continue the experiments in a bigger scale reactor (3 L) (Figure 18).

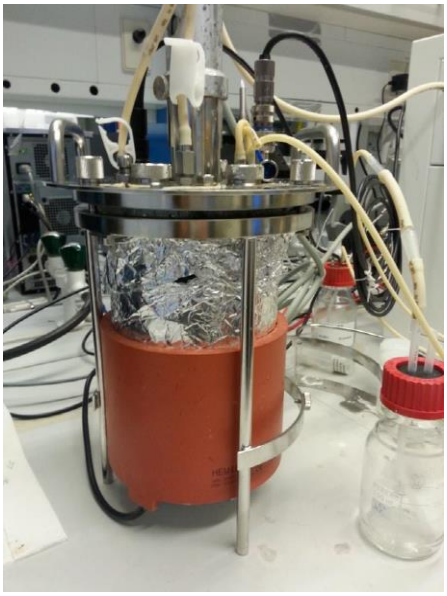

Figure 18: Stirred and aerated 3 L fermenters (Sartorius BIOSTAT APlus, Germany) where the batch fermentation was conducted.

At the end of the experiment the biomass measured was 10.68 ± 1.33 g L^{-1}, representing a biomass yield of 0.40 (g g^{-1} of total sugars (glucose + mannitol)). In particular the protein yield was 0.17 ± 0.06 (g g^{-1} of total sugars (glucose + mannitol)) with an overall productivity of 0.89 ± 0.06 g L^{-1} d^{-1}. The protein content in the microalgae biomass obtained with the proposed approach remarkably increased, from 0.07 ± 0.01 gProtein gDM^{-1} to 0.44 ±

0.04 gProtein gDM^{-1}. Moreover, to evaluate the protein quality and therefore to determine if *C. protothecoides* can be considered a valuable feed supplement, an evaluation of the essential amino acids score (EAA) is crucial. In particular, previous studies demonstrated that a balanced histidine supplementation has a fundamental effect on the growth performance and health of fishes, preventing apoptosis, oxidative damages or bacterial infections (Jiang et al., 2016). With the proposed approach EAA increased by more than 6 times (from 4.86% to 29.40%) with a histidine increase from 0.53 ± 0.27 to 1.52 ± 0.83 mg gDM^{-1}, making *C. protothecoides* an insteresting feed supplement.

3.2.2 Carbon dioxide fixation by microalgae combined with SCP production

The potential of combining *C. sorokiniana* with methanotrophs has been analyzed in Scientific Report I.

Methanotrophs are anaerobic bacteria able to use methane as sole carbon and energy source for their growth, according to the equation reported below (Al Taweel et al., 2012; Strong et al., 2015):

$$\mathbf{CH_4 + 0.104NH_3 + 1.45O_2 \rightarrow 0.52CH_{1.8}O_{0.5}N_{0.2} + 0.48CO_2 + 1.69H_2O} \quad (2)$$

Several industries, such as Norfem Danmark A/S, Calysta (FeedKind), UniBio (UniProtein) and Gaprin (Eckert and Trinh, 2016), use methylotrophs, such as *M. capsulatus,* in the large scale production of SCP using natural gas as carbon source, ammonia to provide N, phosphoric acid as source of P, oxygen and minerals. However, finding more sustainable methane sources to grow *M. capsulatus* is essential.

The utilization of biogas produced though anaerobic digestion of organic matter (composition of 50-75% CH_4 and 25-50% CO_2 and small amounts of other gases such as H_2S, N_2, NH_3 and H_2O) instead of natural gas may increase the sustainability of the process. Moreover, since *M. capsulatus*

requires O_2 for its cultivation, this gas must be externally supplied, besides CH_4. Therefore, the integration of this bacteria with microorganisms, such as microalgae, able to uptake carbon dioxide and emit oxygen through photosynthesis may be an interesting and promising approach for the future.

The aim of the Scientific report I was developing an original and promising strategy that can be integrated in a biorefinery approach. As a result, an innovative bioprocess able to generate a wide range of valuable products and at the same time minimize the residues was developed.

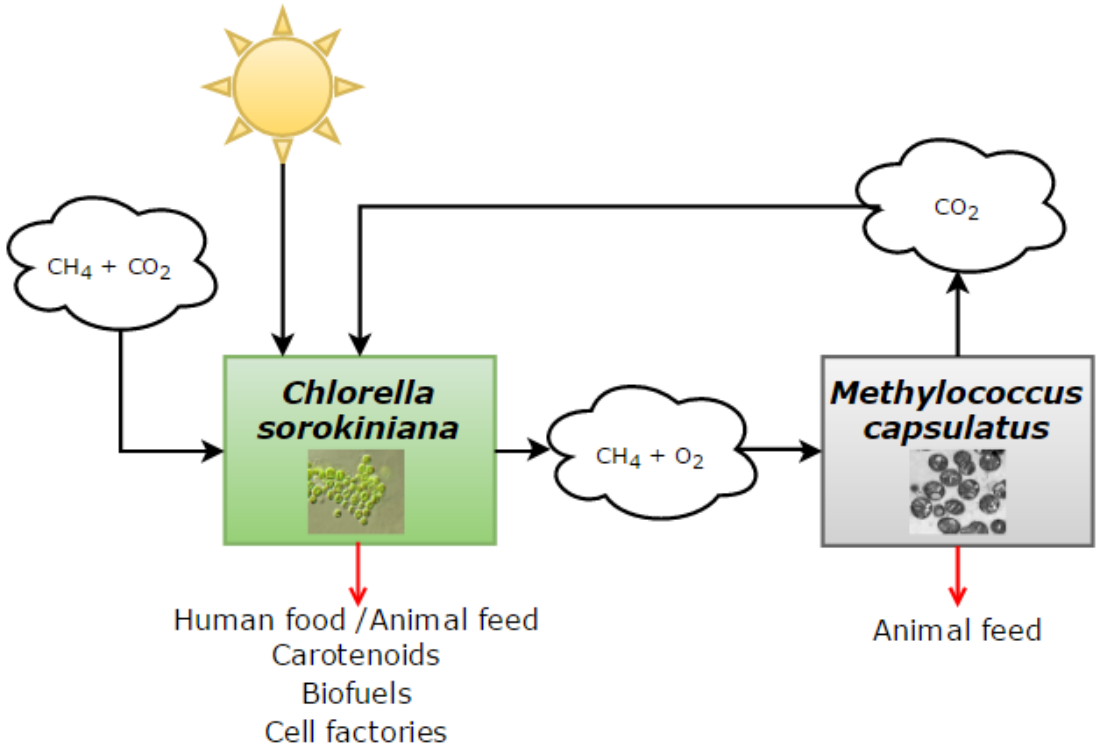

Figure 19: Schematic overview of the process proposed.

Figure 19 shows a schematic overview of the process developed in this study. Microalgae *C. sorokiniana* was grown using biogas (60% CH_4 and 40% CO_2 (v v^{-1})) as a carbon source. The gas coming out from the microalgae growth, which contained CH_4, a reduced amount of CO_2 and O_2 generated through photosynthesis was employed to grow *M. capsulatus*. Finally, the composition of the obtained bacterial and algal biomass was determined in

order to evaluate its nutritional value, in the case of *Methylococcus*, and its potential for the production of high value products, in the case of *Chlorella*.

At the end of the algae experiment a final biomass concentration of 178.33 ± 77.78 mg L^{-1} with a μ_{max} of 3.03 day^{-1}, consistent with the numbers provided by other studies (Lizzul et al., 2014; Van Wagenen et al., 2015), was obtained. The 22.45% of the initial CO_2 is consumed from the biogas and 15-20% O_2 was produced, enabling the utilization of this gas in the growth of *M. capsulatus*.

In the second stage of the process a *M. capsulatus* concentration of 237 mg L^{-1} with protein content in the biomass of 43.34 ± 2.67 %DM was obtained. Moreover the amino acids composition confirmed that the *M. capsulatus* grown with this strategy is a source of all the essential amino acids.

Therefore this approach proved that the integration between microalgae and methanotrophs by means of biogas as carbon source is possible. Moreover the composition of the microalgal and bacterial biomass is extremely interesting, enabling a wide range of potential applications, from feedstock for biodiesel production to cell factories for pharmaceuticals generation, or SCPs for human and animal nutrition.

Hence this strategy represents an interesting process to be implemented in a biorefinery concept.

4 Microalgae biorefinery

In the past decade microalgae have received attention as a potentially interesting feedstock for biofuel production: by thermochemical or biochemical conversion from this specific biomass it is possible to generate biodiesel, ethanol, methane and hydrogen (Demirbas, 2010b). However, due to the high costs, commercial scale production of microalgae-based bioenergy is not sustainable and cannot compete with petroleum-based diesel. Instead, algal biomass is currently utilized for the extraction of high-value products (health, cosmetics, nutraceutical and food) (Acién et al., 2012; Pienkos and Darzins, 2009; Raja et al., 2008). In this scenario, the only realistic way to exploit microalgae for biofuel production is to simultaneously produce other value-added co-products according to a biorefinery strategy (Foley et al., 2011; Olguín, 2012).

4.1 Extraction of lutein from microalga *Chlorella vulgaris*

Lutein is a yellow xanthophyll, member of the carotenoid group. Previous studies demonstrated that balanced lutein assumption is fundamental in preventing diseases in humans and animals (Chiu and Taylor, 2007; Dwyer et al., 2001; Granado et al., 2003; Tominari et al., 2016). Moreover it is used as feed additive in the poultry industry to brighten the color of chicken skin and of egg yolk (Grashorn, 2016).

The main source for extraction and production of lutein are marigold flowers. However, microalgae represent a better candidate because of the higher lutein content and the faster growth rate (Lin et al., 2015). Moreover microalgae can be grown all year on infertile land without any competition with arable crops (Chisti, 2008). However the main limitation in the exploitation of this biomass for lutein production is mainly related to the downstream processes, and in particular to the extraction process.

In Paper IV an improved protocol to extract lutein from the microalga *C. vulgaris* was developed.

The conventional extraction method involves two separated steps: 1) saponification with aqueous KOH, to convert lutein fatty acids esters into free lutein, 2) solvent extraction combined with cell disruption (Cerón et al., 2008; Chan et al., 2013; Li et al., 2002). In the present work, ethanol was used instead of water in the saponification process. As lutein is insoluble in water and 100% soluble in ethanol, employing this solvent the recovery and purity of the pigment were maximized and, at the same time, the extraction time reduced. Moreover, dichloromethane was used in the extraction step and the saponification and the extraction steps, were also conducted simultaneously in order to simplify the entire process.

Figure 20 shows a comparison between the conventional (A) and the novel protocol (B) developed in this study.

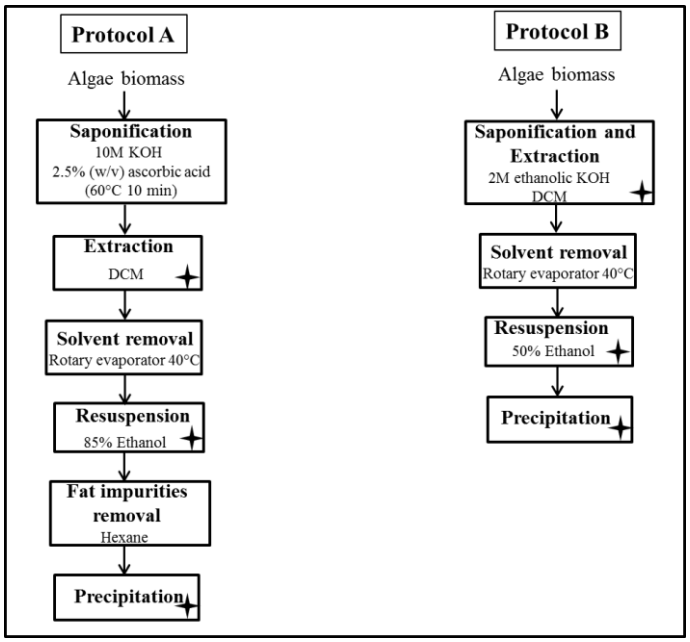

Figure 20: Schematic representation of the conventional (Protocol A) and novel (Protocol B) methods. Only the main steps are included.

The amount of lutein extracted from *C. vulgaris* dried biomass increased more than threefold, from 0.20 ± 0.00 mgLutein gDM^{-1} to 0.69 ± 0.08 mgLutein gDM^{-1}, with a final lutein yield of 20 and 69%, respectively. The reason for such an increase is probably the different solubility of lutein in water and ethanol (0 and 100% respectively). Using ethanol instead of water in the saponification process led to a higher conversion of lutein fatty acid esters into free lutein. Moreover lutein purity was increased from 73.6% to 93.7% by decreasing the ethanol-water ratio from 85% to 50% in the resolubilization step (Figure 21). Indeed, the choice of an optimal ethanol-water ratio, enables the exploitation of the physico-chemical properties of lutein to selectively precipitate this pigment while the others are kept in solution.

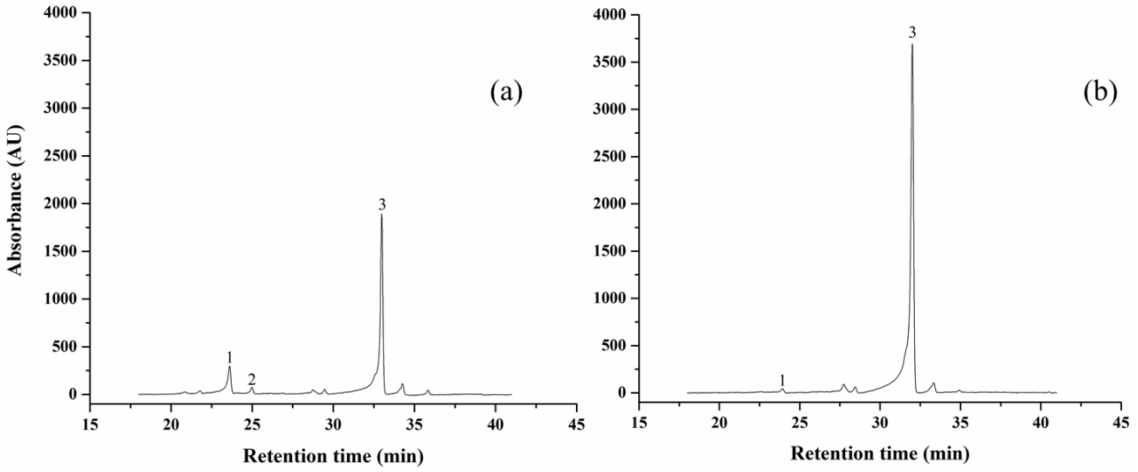

Figure 21: Chromatogram of purified lutein obtained with DCM with the conventional method (a) and with the novel one (b). Peaks: 1, neoxanthin; 2, violaxanthin; 3, lutein.

The novel method was also tested with tetrahydrofuran. The extraction efficiency obtained with this solvent is again better than the conventional protocol; however, dichloromethane was still the solvent of election for this process in terms of quantity (0.69 ± 0.08 mgLutein gDM^{-1} and 0.41 ± 0.00 mgLutein gDM^{-1} with DCM and THF, respectively) and purity (93.7% and 87.4% with DCM and THF, respectively).

4.2 Biorefining of microalgae *Chlorella vulgaris*

In Paper V an innovative biorefinery concept for optimal utilization of microalgal biomass was developed. Lutein and proteins were extracted by the innovative method developed in Paper IV and biogas was generated from the solid and liquid residues after the extractions (Figure 22). All the fractions were characterized in terms of proteins, carbohydrates and lipids (Table 1).

Lutein and proteins were chosen after a preliminary study on the different biomolecules present in the *C. vulgaris* biomass, and after a thorough review of their different extraction protocols. In particular, lutein and proteins have an established and potentially increasing market and, most importantly, it is possible to easily perform the two extraction procedures one after the other, maximizing the recovery of both the products. Indeed, lutein is extracted in hydrophobic conditions, while proteins are extracted exploiting proteins hydrosolubility. Moreover both the protocols are performed in alkaline conditions.

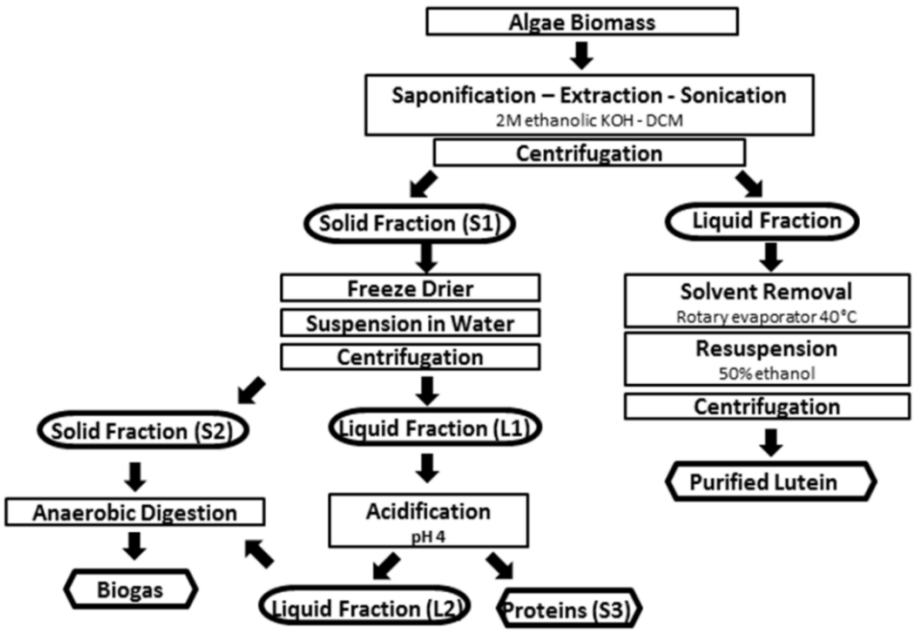

Figure 22: Schematic overview of the biorefinery concept developed for *C. vulgaris*.

Lutein was extracted according to the protocol developed in Paper IV. From the initial algal biomass were extracted 0.8 ± 0.1 mg Lutein gDM^{-1} (approximately 40.8 ± 3.3 mg in total), with a purity of 92.5 ± 1.2%. The lutein yield calculated resulted to be 95%.

The solid residue was then dissolved in water to re-solubilize the proteins contained therein. The proteins are subsequently precipitated by lowering the pH to the value corresponding to the isoelectric point of the majority of the proteins (Chronakis et al., 2000; Gerde et al., 2013). After a preliminary test, to pH 4 corresponded the highest protein precipitation. Then final protein content in the fraction is 82.7 ± 3.1% w $w^{-1}$with a protein yield of 33% if the protein content in the initial algal biomass is considered. However, the protein yield becomes 55% if the protein content of fraction S1 is considered.

Finally biogas was produced from the solid and liquid fractions after protein extraction (S2 and L2) (Figure 22). A methane yield of 372.7 ± 19.0 $NmLCH_4$ gVS^{-1} was recorded, with a biodegradability of 91.1%. Other studies on the same algae reported a methane yield of 286 $NmLCH_4$ gVS^{-1}, with an anaerobic biodegradability of 62% (Dogan-Subasi and Demirer, 2016; Uggetti et al., 2017). Indeed, the main limitation in the anaerobic digestion of microalgal biomass is represented by the presence of low biodegradable macromolecules such as cellulose and hemicellulose in its cell wall (Zamalloa et al., 2011). Previous studies demonstrated that to maximize the organic matter accessible to the anaerobic microorganisms with a consequent higher methane conversion yield a pretreatment step of the algal biomass is crucial (Sialve et al., 2009). Therefore, the extraction steps performed in the previous phases acted as an intensive pretreatment process of the biomass, enabling a much higher biodegrability than the corresponding crude biomass.

The biorefinery scheme presented in Paper V can be considered an example of a successful biorefinery approach for optimal utilization of *C. vulgaris*,

enabling the extraction of two marketable products (lutein and proteins) plus energy (biogas) from the same microalgal biomass.

		Proteins		**Carbohydrates**		**Lipids**	
	DM (g)	% DM	Total amount (g)	% DM	Total amount (g)	% DM	Total amount (g)
Algal biomass	50	49.6	24.8	15.0	7.5	20.0	10.1
S1	37.0 ± 1.3	40.3 ± 1.7	15.1 ± 0.7	14.6 ± 0.6	5.4 ± 0.2	19.3 ± 3.2	7.1 ± 1.2
L1	30.5 ± 1.4	47.7 ± 3.1	14.6 ± 2.5	4.6 ± 1.0	1.4 ± 0.4	15.5 ± 2.0	4.7 ± 0.6
S2	6.5 ± 0.5	7.9 ± 1.9	0.5 ± 0.0	18.2 ± 0.9	1.2 ± 0.3	11.3 ± 3.0	0.7 ± 0.2
L2	20.5 ± 0.9	12.1 ± 0.2	2.5 ± 0.1	6.0 ± 1.3	1.2 ± 0.5	9.3 ± 4.7	2.4 ± 1.2
S3	10.0 ± 1.2	82.7 ± 3.1	8.3 ± 0.3	1.1 ± 0.1	0.1 ± 0.0	24.5 ± 4.2	2.4 ± 0.4

Table 1: Composition (DM (g); %DM) and total amount (g) of each fraction deriving from the different extraction steps (described in Figure 22). The composition of the algal biomass was provided by the manufacturer.

5 Conclusions

This thesis focused on the production of proteins from both macro- and microalgae biomass in a biorefinery perspective. The potential of producing other high value products, such as pigments, was also investigated. Various configurations using different microorganisms, and the integration of macro- and microalgae were analysed. Moreover an evaluation of the effect of the harvesting location and of the season on the macroalgae composition was done. The major contributions resulting from these studies are summarized below:

- The biomethane potential and the total phenolics composition of macroalgae *L. digitata* and *S. latissima* harvested in Danish waters changed seasonally. Moreover also the harvesting location has an impact on the final biomass composition.Samples harvested in summer in Hanstholm represent the best substrate for producing biogas (343.7 ± 21.4 NmLCH4 g VS^{-1}) and extracting total phenolics (0.5 ± 0.0 mgTPC gDM^{-1}).
- Different industrial methods to produce proteins and amino acids were presented and the potential of innovative approaches was highlighted.
- *C. protothecoides* can be grown heterotrophically using *L. digitata* hydrolyzed. Its final composition is rich in proteins (0.44 ± 0.04 gProtein $gDryMatter^{-1}$) and it represents a good SCP to be used as feed supplement.
- A configuration to grow the microalgae *C. sorokininana* together with the microorganism *M. capsulatus* was developed. The final biomass can be used as SCP or as source of high value products such as pigments.
- A novel protocol to extract lutein from microalgae was developed. A lutein yield of 69% was achieved.

- A novel biorefinery strategy based on the microalgae *C. vulgaris* was developed. Proteins and lutein were extracted and biogas was produced from the debris of the process. The potential of microalgae biorefinery for production of high value products was demonstrated.

6 Future perspectives

This project showed that macro- and microalgae can be utilized for the production of both biochemicals and biofuels. However to improve the utilization of this biomass in a more efficient and profitable way further research has to be done. The major suggestions for additional research are reported below:

- Utilization of the post hydrolyses solid residue (PHSR) obtained in the *C. protothecoides* experiment. A full characterization of this fraction has to be done in order to determine which product is the most profitable one in a biorefinery perspective.
- Further development and optimization of the simultaneous production of *C. sorokiniana* and *M. capsulatus*, exploiting the various high value products that can be obtained.
- Utilization of wastewater as nutrient source for the microalgae growth in the *C. sorokiniana* and *M. capsulatus* experiment.
- Life cycle assessment (LCA) on the proposed microalgae biorefinery strategy with the aim of evaluating the environmental sustainability and the economic benefits of the overall process.

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8 Papers

Papers:

I D'Este M., Alvarado-Morales M., Ciofalo A., Angelidaki I., 2017. Macroalgae *Laminaria digitata* and *Saccharina latissima* as potential biomasses for biogas and total phenolics production; focusing on seasonal and spatial variations of the algae. (Accepted for publication in Energy & Fuels)

II D'Este M., Alvarado-Morales M., Angelidaki I., 2016. Amino acids production focusing on the fermentation technologies – A review. (Under review in Biotechnologies Advances)

III D'Este M., Alvarado-Morales M., Angelidaki I., 2017. *Laminaria digitata* as potential carbon source in heterotrophic microalgae cultivation for the production of fish feed supplement. (Under review in Algal Research)

IV D'Este M., De Francisci D., Angelidaki I., 2017. Novel protocol for lutein extraction from microalga *Chlorella vulgaris*. (Under review in Biochemical Engineering Journal)

V De Francisci D., D'Este M., Rasouli Z., Angelidaki I., 2017. Novel biorefinery concept for the extraction of lutein and proteins from microalga *Chlorella vulgaris* and generation of biogas from the residual biomass. (Submitted to Bioresource Technology)

In this online version of the thesis, **paper I-V** are not included but can be obtained from electronic article databases e.g. via www.orbit.dtu.dk or on request from.

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